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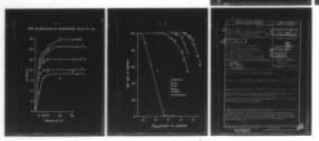

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Protease Inhibitors Suppress Enterotoxin B Formation by *Staphylococcus aureus*

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INTRODUCTION

Our understanding of the details of synthesis and secretion of exoproteins has been significantly deepened since the formulation of the signal hypothesis so elegantly presented by Blobel and Dobberstein (1). The essence of the theory is that translation of messenger RNA yields a polypeptide chain longer than that of the mature, exocellular form of the protein. During translation, a specific protease bound to the membrane cleaves the nascent polypeptide chain, removing a 23-25-amino acid hydrophobic peptide anchor and permitting discharge of the final, mature exoprotein to the exterior. This general mechanism seems to apply to formation of blood proteins (1-3) and hormones (4-8) by eukaryotic cells and to manufacture of alkaline phosphatase (9,10) and penicillinase (11) by bacteria. Aiyappa, Traficante, and Lampen (11) have purified the membrane-bound protease from Bacillus licheniformis which cleaves the nascent polypeptide chain of penicillinase and have concluded from inhibitor studies that it is a serine protease.

Production of enterotoxin B (SEB) by Staphylococcus aureus also involves the synthesis and export, to the exterior medium, of a sizable polypeptide. On the basis of the investigations quoted above, the effect of certain protease inhibitors on production of SEB by both growing and concentrated nongrowing cells of S. aureus was studied.

MATERIALS AND METHODS

The liquid medium used throughout the study was composed of 4% NZ-amine NAK (Humko Sheffield) and 1% yeast extract (Difco) dissolved in 0.067 M phosphate buffer, pH 7.0. For growing cells, tubes containing 5 ml of the NAK medium supplemented with various protease inhibitors

were inoculated with 0.05 ml of an overnight culture of S. aureus strain S-6 in NAK medium. These cultures were incubated at an angle on a shaker at 37°C for 21 hr. The optical densities of a 1:5 dilution (into water) of the 21-hr cultures were determined on a Coleman Jr. spectrophotometer at 600 nm. The remainder of the culture was centrifuged to remove the cells and the supernatant was decanted and stored frozen until assayed for SEB. The amount of SEB (μg/ml) in these samples divided by the optical density of the 1:5 dilution yielded SEB/O.D. values which were employed in the calculations of data presented in Fig. 1.

For concentrated, nongrowing cell studies, 250-ml flasks containing 100 ml of NAK medium were inoculated with 2 ml of an overnight culture of strain S-6 in NAK medium. The flasks were then incubated in a water bath shaker for 5 hr at 37°C. Following this incubation, cells from 100-ml volumes of culture were sedimented in a refrigerated centrifuge and the cells from each were resuspended in 10 ml of ice cold NAK medium containing one of many graded concentrations of a protease inhibitor. One-milliliter samples of these concentrated cell suspensions were then centrifuged to obtain a 0 time value for the amount of SEB present. The cell suspensions were then transferred immediately to a 37°C water bath and 1-ml samples were withdrawn after 10, 20, 30, 60, and 90 min of incubation and centrifuged immediately to remove cells. All supernatants were decanted and stored frozen until assayed for SEB. No change in viable count or optical density could be detected in these suspensions throughout the entire incubation period. The suspensions were three times more concentrated than 21-hr cultures of the organism grown to stationary phase in the NAK medium.

All inhibitors were dissolved in NAK medium and diluted with the same medium to yield the desired inhibitor concentration. For growth studies, the initial inhibitor solution in NAK was sterilized by membrane filtration before use. Phenylmethylsulfonyl fluoride (PMSF), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and para-aminobenzamidine (pABZ) were obtained from Sigma Chemical Company. 1-Chloro-3-tosylamido-7-amino-2-heptanone hydrochloride (TLCK) was purchased from Aldrich Chemical Company. Cerulenin was a product of Makor Chemicals, Ltd. of Jerusalem, Israel. Antipain [(1-carboxy-2-phenylethyl)carbamoyl-L-arginyl-L-valylarginal] was obtained through the courtesy of Dr. Walter Troll, New York University Medical Center (12). None of these inhibitors, at the highest concentration tested, caused a measurable change in pH of the medium.

Assays for SEB were performed by the Oudin tube single diffusion method.

RESULTS AND DISCUSSION

The effects of various protease inhibitors on both final optical density and SEB/O.D. values for growing cultures are presented in Fig. 1. TPCK inhibited equally both growth and SEB formation and no differential suppression of SEB production could be detected. In contrast, pABZ, over a narrow concentration range, strongly inhibited SEB yield without appreciably altering final growth density. Both TLCK and antipain clearly inhibited SEB production over a comparatively broad concentration range without suppressing growth. Antipain had previously been shown (12) to suppress proteases of Escherichia coli over a 10-fold concentration range without detrimental effect on growth of the organism. PMSF, up to

saturation (10^{-3} M), had no effect on either final growth density or SEB formation by this strain of S. aureus (data not shown).

Formation of SEB by concentrated, nongrowing cell suspensions in the presence of inhibitors yielded similar results. The influence of pABZ on SEB production by concentrated suspensions is presented in Fig. 2. Formation of SEB reached a maximum after 30 to 60 min of incubation and the final concentration of SEB formed was clearly suppressed by increasing concentrations of pABZ. The rapid, initial rate of SEB formation was probably also diminished by pABZ, although the data did not clearly demonstrate such an effect. The influence of other inhibitors on SEB production by concentrated cell suspensions is illustrated in Fig. 3. In this figure, the average final concentration of SEB in μg/ml over the range of 30 to 90 min was determined for each concentration of each inhibitor. The results are plotted as the percent of the average final SEB concentration for a control suspension in NAK without inhibitor. TPCK suppressed SEB formation when used at concentrations far above those that completely inhibited growth. Similarly, pABZ severely suppressed SEB production at concentrations up to 50 mM, much higher than the concentration required to prevent growth. TLCK strongly inhibited formation of SEB by these cell suspensions while antipain caused only slight inhibition. Chloramphenicol at 20 μg/ml or more totally inhibited SEB production. This result indicated that SEB formed in this system was derived solely from de novo synthesis of SEB. A similar system and conclusion have been described by Miller and Fung (13). The antibiotic cerulenin had previously been shown to suppress SEB formation by growing cultures of strain S-6 at concentrations (2 to 4 μg/ml) far too low to affect either growth rate or final growth density (14).

Concentrations of up to 100 μg cerulenin per ml (4.7×10^{-4} M), the minimal inhibitory concentration for growth of strain S-6, did not reduce SEB formation by concentrated, nongrowing cell suspensions. Possibly, reduction in SEB production in growing cultures by subinhibitory concentrations of cerulenin resulted from subtle alteration in the phospholipid composition of the cytoplasmic membrane, whereas cerulenin had no effect on SEB formation by concentrated, nongrowing cell suspensions whose membrane composition was probably stable over the 90-min incubation period.

Aiyappa, Traficante, and Lampen (11) reported that purified penicillinase-releasing protease of B. licheniformis was inhibited strongly only by diisopropylfluorophosphate but not by TLCK, TPCK, or pABZ. Formation of SEB by concentrated cell suspensions of S. aureus can be inhibited by these compounds which suggests that a proteolytic cleavage step may well be involved in transport of SEB molecules to the exterior medium.

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Legends

Figure 1.

Suppression by protease inhibitors pABZ, TLCK, TPCK and antipain of SEB formation by growing cultures of S. aureus.

Figure 2.

pABZ suppression of SEB production by concentrated cell suspensions.

Figure 3.

Effect of various inhibitors on SEB formation by concentrated cell suspensions.

FIG. 1

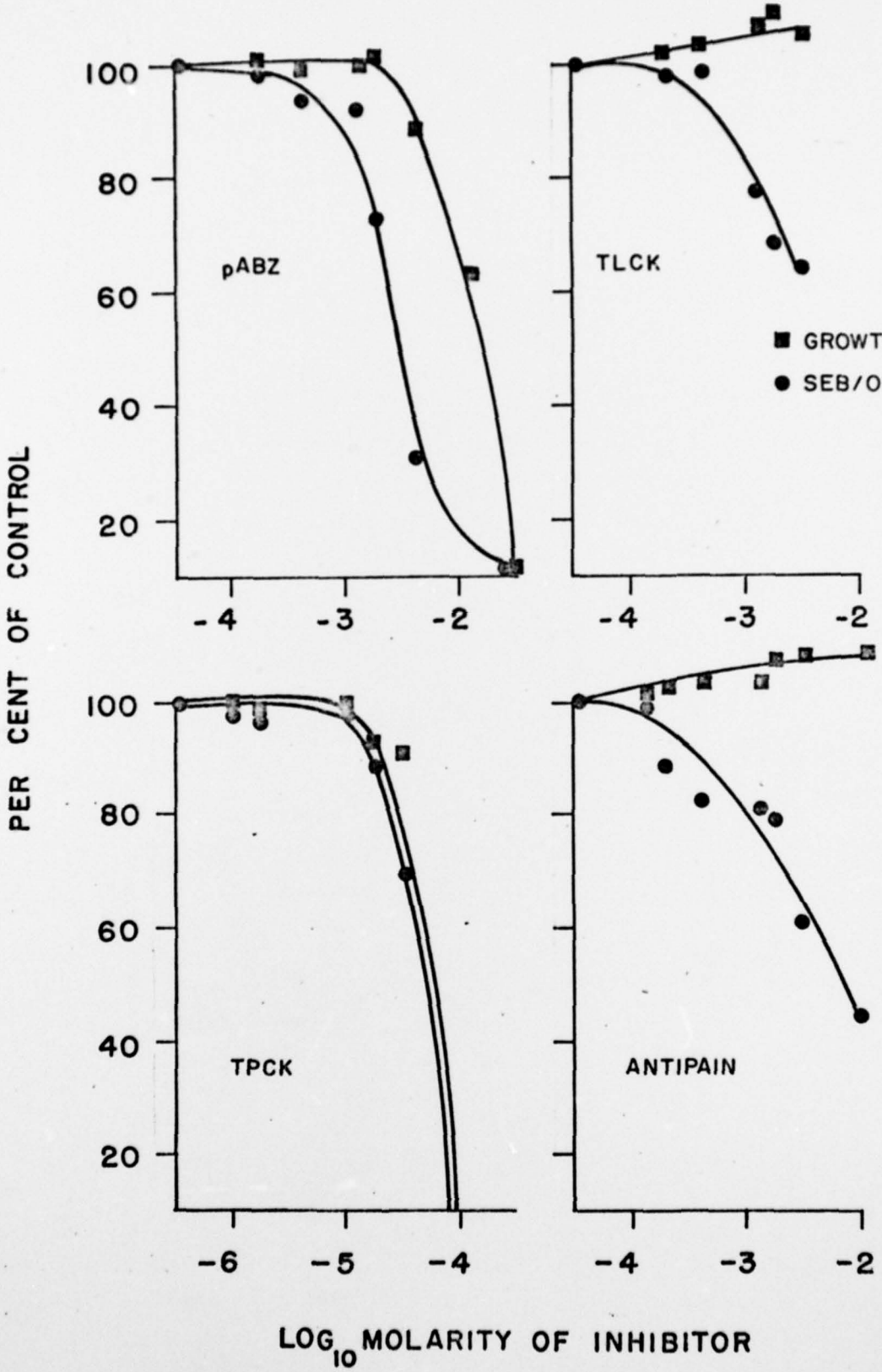

PER CENT OF CONTROL
100
80
60
40
20
pABZ
TLCK
GROWTH
SEB/OD
-4
-3
-2
TPCK
ANTIPAIN
-6
-5
-4
LOG10 MOLARITY OF INHIBITOR

FIG. 2

pABZ ON SEB RELEASE BY CONCENTRATED CELLS OF S-6

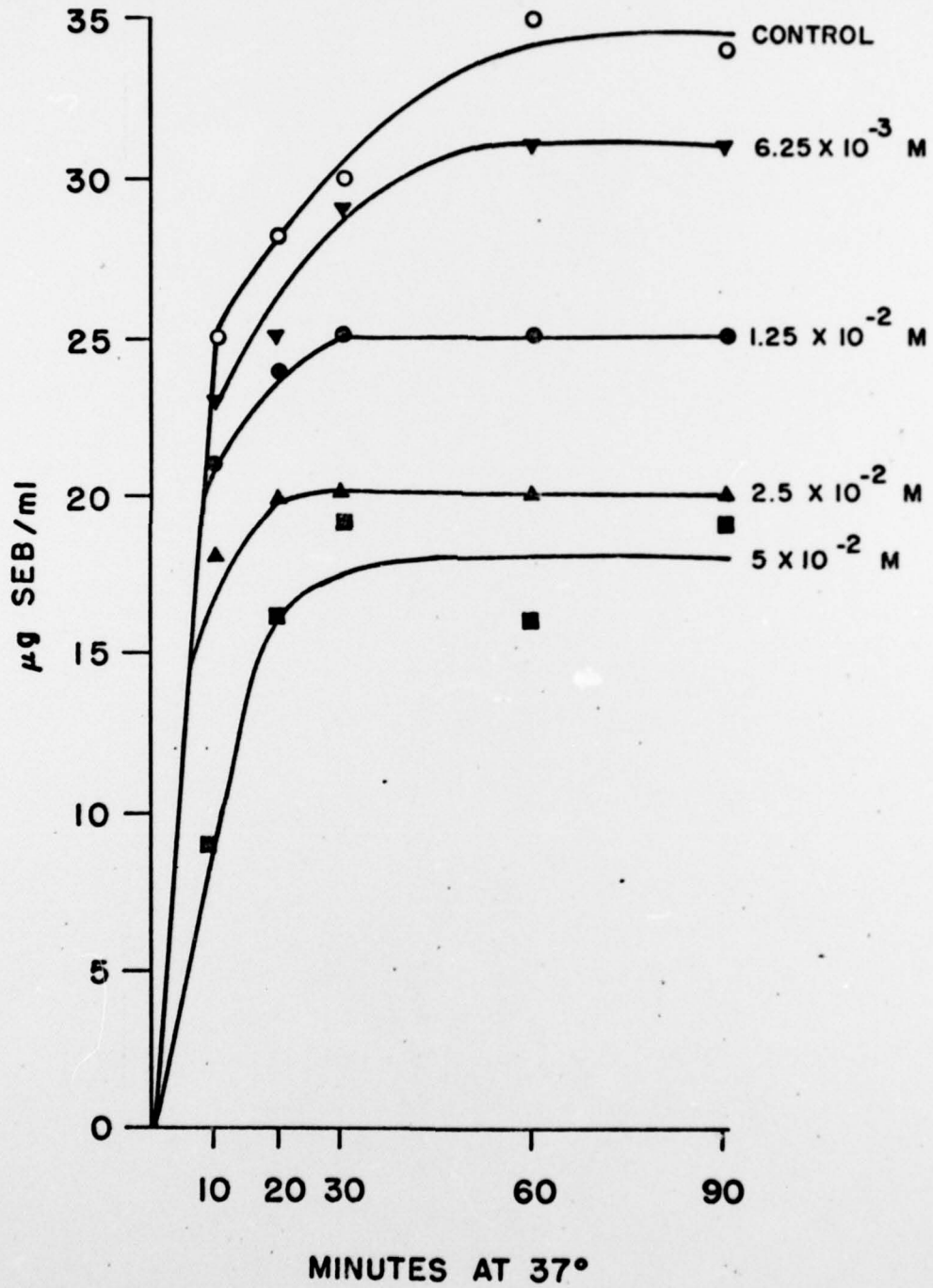
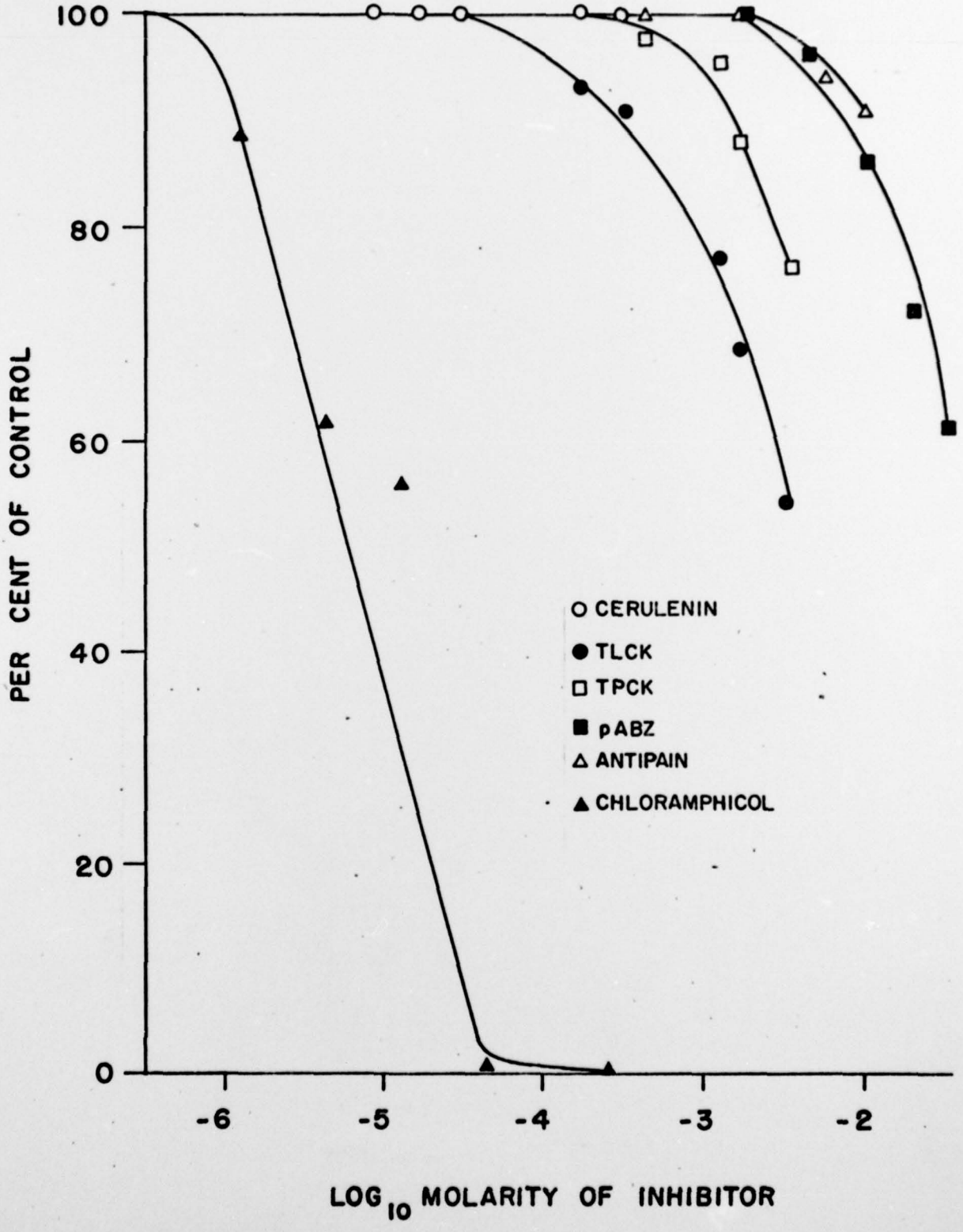


FIG. 3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PER CENT OF CONTROL
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LOG 10 MOLARITY OF INHIBITOR
O CERULENIN
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Certain protease inhibitors depress enterotoxin B production without diminishing growth of the organism. Enterotoxin synthesis by concentrated, resting cells is also decreased by the same inhibitors. The results suggest that a protealytic cleavage step is obligatory for the release of extracellular toxin.

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